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Molecular phylogeny, taxonomy, and evolution of the land snail genus *Pyrenaearia* (Gastropoda, Helicoidea)*

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Abstract: We reconstructed the molecular phylogeny of 78 specimens, including nearly all nominal species of the genus *Pyrenaearia*, and discussed the implications of the molecular phylogeny for species delimitation. The four basal clades obtained by mitochondrial COI and 16S sequences were highly congruent with nuclear ITS markers, shell morphology, and geographic distribution patterns, and were considered different species under the phylogenetic species concept: *Pyrenaearia carascalopsis* (Bourguignat in Fagot, 1884), *P. carascalensis* (Férussac, 1821), *P. parva* Ortiz de Zárate, 1956, and *P. cantabrica* (Hidalgo, 1873). Evidence of reproductive isolation between coexisting species of these basal clades has also been obtained. Because of genetic divergence, together with peculiarities of habitat use, distributional range, and shell morphology, *P. organiaca* (Fagot, 1905) and *P. navasi* (Fagot, 1907) were regarded as valid species. Further subdivisions within *Pyrenaearia* were also considered. Some incongruencies were found between shell morphology and DNA-based taxonomy, including the presence of hairs in adult shells and shell shape adaptations to different altitudes. The deepest split in *Pyrenaearia* involves four different lineages and represents an ancient event, occurring during the Pleistocene or even the Pliocene. Subsequent *Pyrenaearia* speciation within the four main groups has been a very recent process, and it is hypothesized to have occurred during Pleistocene cycles of climatic cooling and warming. Chronologically, a third speciation process is occurring from the Würm de-glaciation to the present time. All these processes included allopatric speciation of the high-altitude taxa during warmer interglacial periods, but parapatric or peripatric speciation events could also be involved. Based on the present day distribution of the species and their different altitudinal preferences, the whole genus could constitute a good model to investigate the evolutionary processes that created this diversity. It could also be an excellent group of closely related taxa for the study of the effect of Plio-Pleistocene climate changes on species distribution, population structure, speciation processes, secondary contacts, and passive dispersal.

Key words: DNA, systematics, distribution, land snails

The genus *Pyrenaearia* Hesse, 1921 is endemic in the north Iberian Peninsula and southern France, where it occurs in the Cantabrian Mountains and the Pyrenees (Fig. 1). Species of this genus generally live at high altitudes in open limestone habitats and occupy very restricted geographical areas (Altonaga *et al.* 1994, Puente 1994). They usually occur on rock walls (Ortiz de Zárate 1956), but above 2000 m they live predominantly under stones (Gómez-Moliner, pers. obs.). Based on distributional patterns, the 16 currently recognized nominal species in the genus can be divided in six species groups. (1) Six morphospecies are endemic in the Cantabrian Mountains, where *Pyrenaearia cantabrica* (Hidalgo, 1873) has a relatively wide distribution, while *P. covadongae* Ortiz de Zárate, 1956, *P. daanidentata* Raven, 1988, *P. oberthueri* (Ancey, 1884), *P. poncebensis* Ortiz de Zárate, 1956, and *P. schaufussi* (Kobelt in Rossmässler, 1876) are confined to the Picos de Europa Massif. (2) *Pyrenaearia velascoi* (Hidalgo, 1867) is restricted to the Basque Mountains. (3) Another five morphospecies are endemic in the central and western Pyrenees with *P. carascalensis* (Férussac, 1821) showing the widest distribution, whereas *P. carascalopsis* (Bourguignat in Fagot, 1884), *P. cotiellae* (Fagot, 1906), and *P. esserana* (Bourguignat in Fagot, 1888) are limited to small areas in the central Pyrenees. *Pyrenaearia transfuga* (Fagot 1885) is even restricted to a single locality (Escot) in the Aspe valley at the northern slope of the western Pyrenees. (4) Two species are confined to the eastern pre-Pyrenees: *P. organiaca* (Fagot, 1905) and *P. parva* Ortiz de Zárate, 1956. (5) *Pyrenaearia molae* Haas, 1924 lives in the mountains of Tarragona (Catalonia). (6) Finally, *P. navasi* (Fagot, 1907) is

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Figure 1. Map showing collection sites of the specimens used in this study. The small box at the lower left corner indicates the distribution of the genus *Pyrenaearia* in black.

the only species living south of the Ebro river valley, where it is confined to Mount Moncayo. Nevertheless, Prieto (1991) recognized two subspecies within *P. navasi*: *P. n. navasi* and *P. n. sylvatica*.

Most *Pyrenaearia* spp. are considered as sub-alpine organisms (Ortiz de Zárate 1956, Prieto 1986, Puente 1994), including *Pyrenaearia daanidentata*, *P. oberthueri*, *P. velascoi*, *P. carascalensis*, *P. carascalopsis*, *P. cotiellae*, *P. esserana*, *P. parva*, and *P. navasi navasi*. These cold-adapted species have allopatric distributions, with often strongly isolated populations inhabiting the highest mountains, while other taxa are confined to the valleys (*P. covadongae*, *P. poncebensis*, *P. schaufussi*, *P. transfuga*, *P. organiaca* and *P. navasi sylvatica*). Only *P. cantabrica* shows a wide altitudinal range from 200 m to 2600 m above sea level.

The taxonomic validity of the 16 nominal *Pyrenaearia* species and subspecies is not clear. Most species show no diagnostic differences in their reproductive apparatus although characters such as body color and relative sizes of structures in the reproductive system have been used to distinguish a few taxa (Ortiz de Zárate 1956, Puente 1994). Thus, taxon identification in *Pyrenaearia* is based mostly on shell characters, including shell size, shape and color, peristome morphology, and the presence of hairs on the shell surface (Ortiz de Zárate 1956, Puente 1994). However, shell morphology can be profoundly influenced by environmental cues, including altitude (Raven 1988), and may be subject to parallel or convergent evolution, resulting sometimes in arbitrary classifications and incorrect species determination (*e.g.*, Giusti and Manganelli 1992, Uit de Weerd *et al.* 2004).

Delimiting species and reconstructing their phylogenetic relationships are the two major goals of systematics (Wiens and Penkrot 2002). In this context, recent developments in DNA-based taxonomy aimed at defining species using the evolutionary and phylogenetic species concepts (Simpson 1961, Wiley 1978, Cracraft 1983, Coyne and Orr 2004) are extremely useful in validating morphospecies in several animal groups (Vogler and Monaghan 2007). Molecular genetic tools have also become very popular for reconstructing phylogenetic relationships in animal species (Avise 2000) and for identifying speciation processes, analyzing population structure, and inferring phylogeographic patterns.

The present work is the first molecular phylogenetic study of the genus *Pyrenaearia*. We used DNA sequence data to (1) assess the validity of the 16 nominal *Pyrenaearia* species under the morphological, phylogenetic, and biological species concepts, (2) reconstruct their phylogenetic relationships, and (3) evaluate the geographic limits of the different lineages and taxa. Based on this information we develop some tentative speciation scenarios in relation to the Plio-Pleistocene climatic changes.

MATERIALS AND METHODS

Seventy-eight specimens of *Pyrenaearia* were included in this study with *Hygromia limbata* (Draparnaud, 1805) and *Iberus gualtieramus* (Linnaeus, 1758) as outgroups. Locality and sample data are provided (Fig. 1, Appendix 1). Whenever possible, topotypes were included in the analysis. Freshly collected animals were kept frozen at -20 °C until analysis. Collection specimens were preserved in 70% ethanol.

Individual DNA was extracted from foot muscle (15 to 20 mg), using the DNAeasy Tissue kit (QIAGEN). In some cases tissue was removed, squashed, and homogenized in sucrose buffer in order to eliminate excess mucopolysaccharides (Moorsel *et al.* 2000).

Polymerase chain reaction (PCR) was used to amplify two mitochondrial gene fragments, cytochrome oxidase subunit I (COI) and 16S rRNA (16S), and one complete nuclear gene, the ribosomal internal transcribed spacer 1 (ITS-1).

The COI fragment was initially amplified using the primers described by Folmer *et al.* (1994) [LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′), HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAATCA-3′)]. The amplicons were sequenced for various *Pyrenaearia* species and a

new specific forward primer was designed for this study to amplify a shorter fragment of about 500 base pair (bp) [COIP1F (5′-TAATGTTGTAGTTACTGC-3′)]. The COIP1F primer was used together with the HCO 2198 reverse primer of Folmer *et al.* (1994). The 16S fragment was amplified by the primers of Palumbi *et al.* (1991) [16SarL (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SbrH (5′-CCGGTCTGAACTCAGATCACGT-3′)]. The ITS-1 marker was amplified using the primers ITS1L (5′-TCCGTAGGTGAACCTGCGGAAGGAT-3′) and 58C (5′-TGCGTTCAAGATATCGATGTTCAA-3′) (Hillis and Dixon 1991). Amplicons were sequenced using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) run on an ABI PRISM Model 3100 Avant Genetic Analyzer. The sequences were deposited in GenBank (accession numbers in Appendix 1). All sequences were aligned with Clustal X version 1.8 (Thompson *et al.* 1997) with default settings and they were refined manually where necessary.

Phylogenetic analyses using PAUP* 4.0b 10(PPC) (Swofford 2002) were performed for the three DNA fragments independently as well as for the combined data set. Gaps were treated as missing data. The best model of sequence evolution was selected using the Akaike information criterion (AIC) implemented in Modeltest v3.06 (Posada and Crandall 1998).

A heuristic search was performed for the maximum parsimony (MP) analyses, with ten random-addition replicates, using the tree bisection reconnection (TBR) option generating multiples trees to determine the most parsimonious solution. Weights of transversions (Tv) and transitions (Ts) varied depending on the fragment and were estimated by maximum likelihood. The weighting scheme was 4:1 for COI and 1:1 for 16S and ITS-1.

Neighbor-joining (NJ) (Saitou and Nei 1987) trees were constructed using the GTR+I+G model (Rodríguez *et al.* 1990) for all data matrices, according to Modeltest results. Uncorrected pairwise "p" distances were also calculated for the four data sets (see Table 1). For both the MP and NJ analyses, bootstrap (BS) confidence estimates were based on 1000 replicates (Felsenstein 1985). Any BS values higher that 70% were considered as providing strong support (Hillis and Bull 1993).

Bayesian analyses (BA) were performed using the MrBayes v3.0 package (Huelsenbeck and Ronquist 2001). The GTR model was used for the four data matrices, and rate variation across sites was modeled using a gamma distribution for COI data set and equal distribution for 16S, ITS-1, and combined data sets, with a proportion of the sites being variants. The Markov Chain Monte Carlo (MCMC) search was run with four chains for 2 million generations, with trees being sampled every 100 generations (the first 2000 trees were discarded as "burn-in"). In the combined analyses, variation was partitioned among genes.

Table 1. Molecular diversity indices for the different clades of *Pyrenaearia* for COI (first line), 16S rRNA (second line), and ITS-1 (third line). Values of number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and mean number of nucleotide differences (k) are reported.

	CLADE 1	CLADE 2	CLADE 3	CLADE 4
H	4	3	20	27
	3	4	23	24
	3	1	8	5
h	0.9	0.833	0.965	0.986
	0.7	1.0	0.953	0.975
	0.7	0	0.713	0.321
π	0.021	0.004	0.03	0.027
	0.008	0.006	0.027	0.016
	0.005	0	0.002	0.002
k	9.4	2.0	13.361	12.068
	3.1	2.5	10.729	6.383
	3.4	0	1.112	1.444

RESULTS

Sequence characteristics and genetic divergences

The number of mtDNA haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and number of nucleotide differences (k) were calculated for each gene fragment and for the combined data set (Table 1).

The aligned COI fragment comprised 443 base pairs (bp). Sequences were A:T rich (74.17%) with nucleotide composition of: T (43.72%), C (11.2%), A (30.45%), and G (14.63%). One hundred and thirteen (25.51%) positions were parsimony informative. Using the *Drosophila* mitochondrial genetic code, 100 (68.03%) of the 147 amino acids had synonymous nucleotide substitutions and 47 (31.97%) had no substitutions.

The aligned 16S fragment consisted of 408 bp. The sequences were A:T rich (69.84%), with nucleotide compositions of: T (33.78%), C (12.54%), A (36.06%), and G (17.62%). Eighty-nine (21.81%) positions were parsimony informative.

The aligned ITS-1 gene comprised 692 characters. The proportion of A:T (40.95%) was lower, with nucleotide compositions of: T (22.18%), C (27.12%), A (18.77%), and G (31.93%). Seventeen (2.46%) positions were parsimony informative.

The combined data set of the COI, 16S, and ITS-1 analyses consisted of 1543 bp, 1069 of which (66.69%) were invariable, while 284 (18.4%) were variable but parsimony uninformative and 230 (14.91%) were parsimony informative.

Phylogenetic analyses

The four NJ trees had nearly identical topologies (Figs. 2-4) and were also almost identical to the MP and BA

topologies (not shown). The p-distances based on COI were larger than those based on 16S. All haplotypes were grouped within four basal clades. Average p-distances between these four clades were higher than 8.5% and 4.5% for COI and 16S, respectively (Table 2). The mean values of the average pairwise p-distances between a basal clade and the other three (Table 2) were very similar for each mtDNA gene fragment (8.6-9.8% for COI; 5.2-6.9% for 16S).

ITS-1 was nearly 10× more conservative than COI and 16S (Table 2) and also recovered four major groups, the p-distances between which amounted to a maximum of 1.1%. The four ITS-1 groups corresponded to mtDNA clades, with clade 3a joined together with the other clade 3 subgroups in a common polytomy.

The combined data set of the three genes, COI, 16S, and ITS-1, yielded a well-supported topology (Fig. 4). Basal nodes were not resolved but the monophyly of each of the four basal clades was strongly supported by bootstrap values (>95%), except for clades 3 and 4 in the MP analysis. The four basal clades joined the same populations in the COI, ITS-1, and the concatenated phylogenetic analyses (Figs. 2-4). Clade 3a was recovered as an independent lineage in the 16S tree. These results suggest confidence in the phylogeny obtained for *Pyrenaearia*, since the separate and combined analyses of COI, 16S, and ITS-1 did not yield conflicting results. Therefore, we will use the combined phylogeny for further discussion.

Clade 1 comprised the haplotypes of *Pyrenaearia carascalopsis* and *P. esserana*, with the former constituting a paraphyletic group. Clade 2 comprised the haplotypes of *P. parva* from two localities, including topotypes. Clade 3 grouped *P. carascalensis* together with *P. cotiellae*, *P. molae*, *P. navasi*, *P. organiaca*, *P. transfuga* and *P. velascoi*. *Pyrenaearia organiaca* was the basal group of clade 3 and showed the greatest divergence within it (mean values of the average p-distances of 7.7% and 6.9% for COI and 16S, respectively) (Table 3). *Pyrenaearia navasi* was the sister group of clades 3c-f. The mean values of the average p-distances among clade 3 subgroups (3b-f) were below 5.0%, 3.6%, and 0.3% for COI, 16S and ITS-1, respectively (Table 3). The analysis fully resolved the phylogenetic position of *P. organiaca* and *P. navasi*. Nevertheless, the phylogenetic relationships of *P. carascalensis*, *P. cotiellae*, and *P. molae* could not be resolved.

The monophyly of *P. velascoi* was well supported (BS >85%) and this nominal species was grouped in subclade 3f with *P. transfuga* and with the populations of *P. carascalensis* from the West-Pyrenees. *Pyrenaearia transfuga* and *P. carascalensis* appeared in an unresolved polytomy. Excluding *P. velascoi*, all the haplotypes of the Cantabrian *Pyrenaearia* populations were grouped in clade 4, which thus comprised *P. cantabrica*, *P. oberthueri*, *P. daanidentata*, *P. poncebensis*, and *P. schaufussi*.

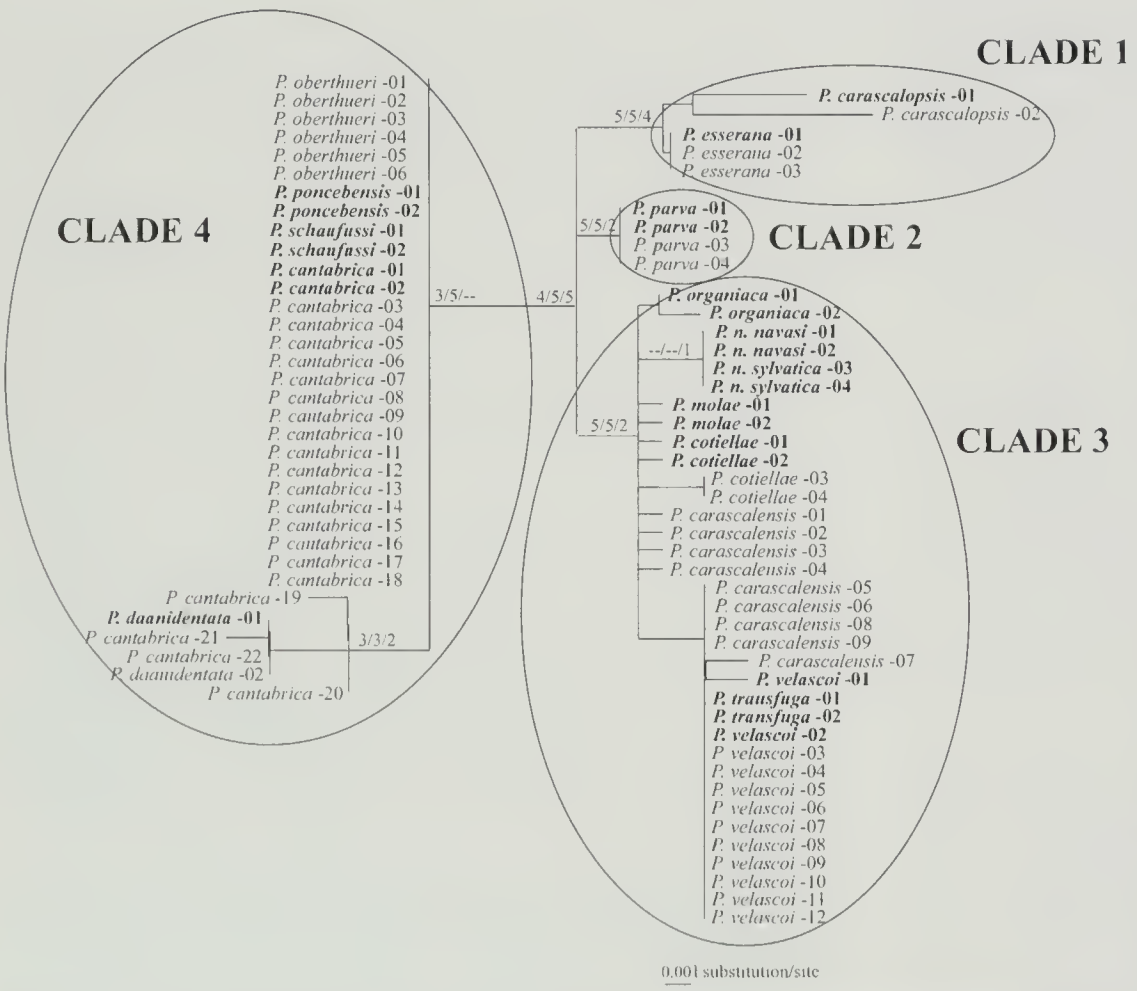

Figure 2. Unrooted NJ tree of nuclear ITS-1 from *Pyrenaearia* specimens. Bootstrap values under NJ, MP, and posterior probabilities under MB are shown at each node (NJ/MP/MB) when >70%. Number codes indicate bootstrap values and posterior probabilities: 1, 100%; 2, 99-95%; 3, 94-90%; 4, 89-80%; and 5, 79-65%.

DISCUSSION

Species delineation and taxonomy

The present study is the first attempt ever to determine species delineation of *Pyrenaearia* using DNA-based systematics. All haplotypes were unambiguously grouped within four main clades. The phylogeny of *Pyrenaearia* was highly consistent with the current geographical distribution of the clades. According to the explicit tree-based species delimitation protocol recommended by Wiens and Penkrot (2002) and the geographic distribution, we conclude that the four clades should

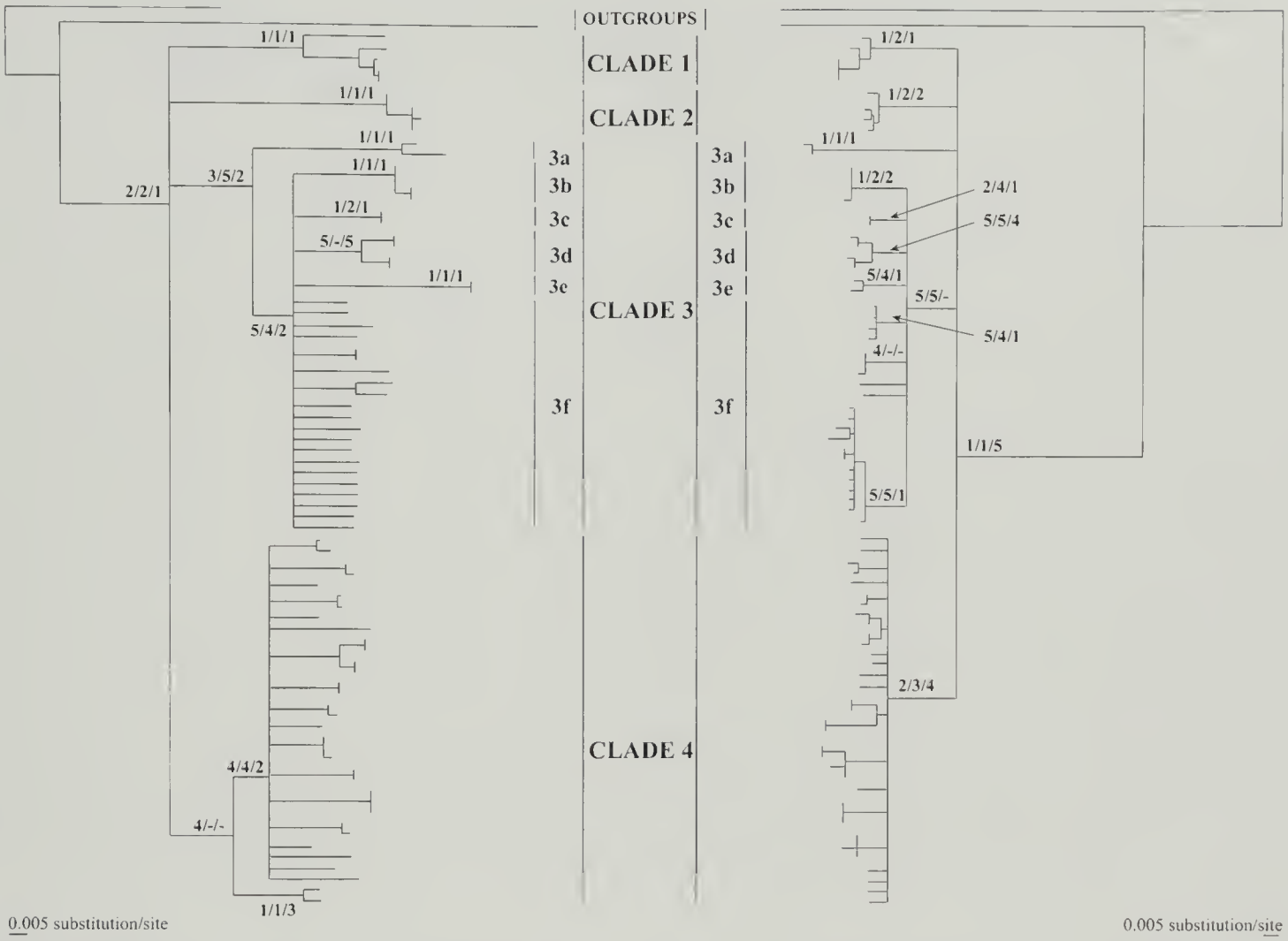

Figure 3. Molecular phylogenies of COI (left) and 16S rRNA (right) by NJ analyses. Bootstrap values under NJ, MP, and posterior probabilities under MB, are given at each node (NJ/MP/MB) when >70%. Bootstrap values are indicated by number codes (see legend of Fig. 2).

be considered different species under the phylogenetic species concept. With the exception of clades 3f and 4, all the clades are allopatric and, consequently, their reproductive isolation could not be tested. The species inhabiting the Pyrenees, the eastern pre-Pyrenees, the Tarragona, and the Moncayo mountains involved three basal clades.

Pyrenaearia carascalopsis and *P. esserana* formed a strongly supported phylogroup (clade 1). Both nominal species were not reciprocally monophyletic suggesting that they constitute a single taxon instead of two different species as proposed by Fagot (1906). In addition, some authors placed both nominal species in the synonym *P. carascalensis* (Germain 1930, Puente 1994). Yet our analyses showed that this opinion cannot be supported, and we instead agree with Prieto (1986) who joined *P. carascalopsis* and *P. esserana* in a single taxon because both taxa have neither well-defined morphological differences, nor particular geographical ranges.

Pyrenaearia parva (clade 2) is a valid morphospecies based on differences in shell morphology and body color (Ortiz de Zárate 1956, Prieto 1986, Puente 1994). It constitutes a well-defined clade that will not be discussed further. In this case, morphological and molecular evidence concerning the species status of *P. parva* were concordant.

Clade 3 joined seven nominal species. *Pyrenaearia organiaca* and *P. navasi* were recovered as two well-supported clades. Both morphospecies are very different from other *Pyrenaearia* taxa in terms of shell shape and geographic distribution. *Pyrenaearia organiaca* lives at low altitudes in the eastern pre-Pyrenees and its shell is very different in size, color, and sculpture (Ortíz de Zárate 1956, Bech 1990, Puente 1994). *Pyrenaearia navasi* is the only *Pyrenaearia* taxon occurring south of the Ebro valley and it also has a unique shell shape, shell color, and fragility (Prieto 1991). Therefore, we suggest that *P. organiaca* and *P. navasi* should be treated as valid species, based on phylogenetic and morphological criteria. The four *P. navasi* haplotypes form an unresolved intraspecific polytomy, thus eventual subspecific categories cannot be recognized. *Pyrenaearia carascalensis*, *P. molae*, *P. cotiellae*, *P. velascoi*, and *P. transfuga* formed a species complex (referred to as *P. carascalensis s.l.*) whose taxonomy can only be resolved after more intensive sampling and/or by screening more-sensitive markers. *Pyrenaearia cotiellae*,

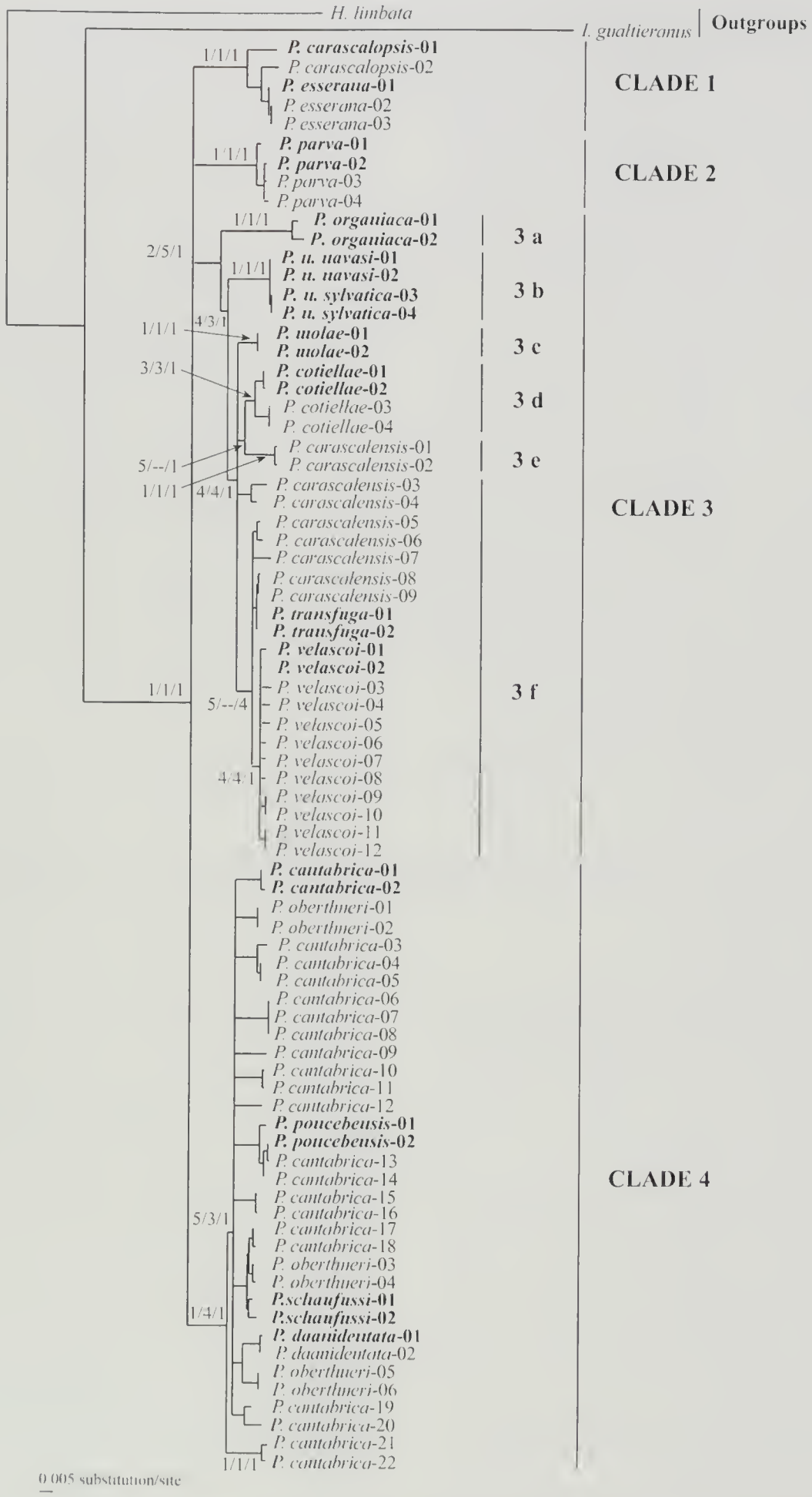

Figure 4. Molecular phylogeny of *Pyrenaearia* as revealed by (NJ) analysis for the combined data set of the COI, 16S rRNA, and ITS-1 genes. Bootstrap values under NJ, MP, and posterior probabilities under MB are shown at each node (NJ/MP/MB) when >70%. Topotype specimens are indicated in bold. Bootstrap values are indicated by number codes (see legend of Fig. 2).

Table 2. Uncorrected average (± *SD*) p-distances of COI (first line), 16S (second line), and ITS-1 (third line) within and among clades. Mean values of the average p-distances between a clade and the other three are also indicated.

	CLADE 1	CLADE 2	CLADE 3	CLADE 4
	2.12 %			
CLADE 1	0.77 %			
	0.52 %			
	11.36 % ± 0.0238	0.45 %		
CLADE 2	6.26 % ± 0.003	0.63 %		
	0.72 % ± 0.0035	0 %		
	10.48 % ± 0.0067	9.50 % ± 0.0066	3.01 %	
CLADE 3	7.23 % ± 0.0048	5.76 % ± 0.0041	2.82 %	
	1.07 % ± 0.0030	0.70 % ± 0.0008	0.16 %	
	8.94 % ± 0.0053	9.06 % ± 0.0053	8.50 % ± 0.0090	2.72 %
CLADE 4	6.72 % ± 0.0038	4.56 % ± 0.0044	6.05 % ± 0.0061	1.6 %
	1.24 % ± 0.0038	0.81 % ± 0.0016	1.08 % ± 0.0018	0.21 %
	9.81 % ± 0.0116	9.42 % ± 0.0095	8.79 % ± 0.011	8.56 % ± 0.0087
Mean values	6.94 % ± 0.0052	5.24 % ± 0.0077	6.17 % ± 0.007	5.99 % ± 0.0076
	1.13 % ± 0.0036	0.76 % ± 0.0016	1.05 % ± 0.0022	1.08 % ± 0.0023

Table 3. Uncorrected average (± *SD*) p-distances of COI (first line), 16S rRNA (second line), and ITS1 (third line) within and among clade 3 subgroups. Mean values of the average p-distances between clade 3 subgroups and the other five are also indicated.

	CLADE 3a	CLADE 3b	CLADE 3c	CLADE 3d	CLADE 3e	CLADE 3f
	1.58 %					
CLADE 3a	0.25 %					
	0.15 %					
	8.92 % ± 0.0040	0.3 %				
CLADE 3b	5.98 % ± 0.0017	0.13 %				
	0.37 % ± 0.0007	0 % ± 0.0000				
	7.44 % ± 0.0026	5.64 % ± 0.0024	0 %			
CLADE 3c	6.16 % ± 0.0014	2.83 % ± 0.0012	0 %			
	0.22 % ± 0.0008	0.15 % ± 0.000	0 %			
	7.90 % ± 0.0034	5.42 % ± 0.0033	3.5 % ± 0.0012	1.05 %		
CLADE 3d	6.66 % ± 0.0023	3.21 % ± 0.0031	2.39 % ± 0.003	0.92 %		
	0.29 % ± 0.0011	0.22 % ± 0.0008	0.07 % ± 0.0008	0.1 %		
	8.80 % ± 0.000	7.45 % ± 0.0024	5.19 % ± 0.000	4.4 % ± 0.0012	0 %	
CLADE 3e	6.18 % ± 0.0032	3.85 % ± 0.0012	1.76 % ± 0.000	1.95 % ± 0.0026	0.76 %	
	0.22 % ± 0.0009	0.15 % ± 0.000	0 % ± 0.000	0.07 % ± 0.0008	0 %	
	7.36 % ± 0.0050	4.51 % ± 0.0048	2.44 % ± 0.0045	1.97 % ± 0.0036	4.01 % ± 0.0047	0.88 %
CLADE 3f	7.31 % ± 0.0057	3.63 % ± 0.0032	2.63 % ± 0.0043	3.21 % ± 0.004	2.95 % ± 0.0033	1.24 %
	0.37 % ± 0.0010	0.3 % ± 0.0006	0.15 % ± 0.0006	0.22 % ± 0.0006	0.15 % ± 0.0006	0.06 %
	7.71 % ± 0.0073	4.92 % ± 0.009	3.17 % ± 0.0126	2.72 % ± 0.0132	4.58 % ± 0.0122	3.24 % ± 0.0121
Mean values	6.93 % ± 0.0071	3.53 % ± 0.0038	2.57 % ± 0.0043	3.06 % ± 0.0052	2.86 % ± 0.0063	3.21 % ± 0.0051
	0.34 % ± 0.0011	0.27 % ± 0.0008	0.13 % ± 0.0007	0.2 % ± 0.001	0.13 % ± 0.0007	0.22 % ± 0.0009

P. molae, and *P. velascoi* are monophyletic lineages and constitute three phylogeographic units in the sense of Avise (1989). Therefore, we suggest that they should maintain their nomenclatorial identity within the *P. carascalensis s.l.* group. Definition of other phylogeographic units within *P. carascalensis s.l.* would require a wider geographic sampling.

Finally, clade 4 comprised five nominal species from the Cantabrian mountains: *Pyrenaearia cantabrica*, *P.*

daanidentata, *P. obertlueri*, *P. poncebensis*, and *P. schaufussi*. Haplotypes of *P. poncebensis* and *P. schaufussi* topotypes were assigned to the *P. cantabrica* haplogroup. Thus, we include both names in the synonymy of *P. cantabrica*. Moreover, the morphological distinction between *P. schaufussi* and *P. cantabrica* has never been well documented (Azpeitia 1925, Ortiz de Zárate 1956, Prieto 1986, Puente 1994) and both taxa were usually defined by their geographical distribution (Altonaga *et al.* 1994), with *P. cantabrica* living in the West-Cantabrian mountains and *P. schaufussi* in the East. The morphospecies *P. poncebensis* was defined by the presence of periostracal hairs on adult shells (Ortiz de Zárate 1956). Haired *Pyrenaearia* specimens have been observed in several localities, interspersed between the localities of *P. cantabrica* (Puente 1994) and recently, we have observed haired adult shells even in the locus typicus of *P. cantabrica*. Clearly, our molecular results suggest that the presence of hairs in adult shells is not diagnostic in *Pyreanaearia* but rather represents a microhabitat adaptation that probably allows snails to retain water from fog in dry places. This hypothesis is based on the observation that haired *P. poncebensis* live under overhanging rocks in the locus typicus while smooth, hairless shells occur on neighboring vertical walls.

Pyrenaearia oberthueri did not represent a monophyletic group within clade 4. This taxon possesses an altitudinal cline in shell size and shape (Ortiz de Zárate 1956), with small, conical forms at higher altitudes (*oberthueri* morph) and larger, flattened shells in the valleys (*cantabrica* morph). Both morphs possess a bluish shell color with a brownish-red peristome (Ortiz de Zárate 1956). As such, *P. oberthueri* seems to be merely a high-altitude form of *P. cantabrica*, living above 1500 m in the Central Massif of Picos de Europa. The bluish color of the valley morph shells is probably a local variation of *P. cantabrica*. More studies are needed to survey and understand this clinal variation and to uncover additional geographical structure in populations (*e.g.*, haplotypes from Santa Ana mountains).

Pyrenaearia daanidentata is the only monophyletic morphospecies in clade 4. It is restricted to the highest altitudes (>2000 m) of the West Massif of Picos de Europa (Peña Santa) and its shell differs strongly from that of *P. cantabrica* by growing slower, being smaller and possessing two strong teeth in the peristome (Raven 1988). Based on the phylogenetic results, together with morphology and geographic distribution, *P. daanidentata* should retain its taxonomic identity. Whether this taxon has a specific or subspecific status requires further research.

Interestingly, *Pyrenaearia cantabrica* and *P. velascoi* (clades 4 and 3f, respectively) coexist above 1300 m in three mountains of the Basque country: Gorbea [Aldamin (30TWN1865), Peña Lekanda (30TWN1668)] and Beriain (30TWN8349) (Prieto 1986; present work). Yet, without exception, the haplotypes of *P. velascoi* and *P. cantabrica* from these areas were assigned to clades 3f and 4, respectively. These results, together with the absence of intermediate shell forms in these localities (Gómez-Moliner, pers. obs.), suggest that both taxa do not hybridize, so that *P. cantabrica* and *P. carascalensis s.l.* (including the nominal species *P. transfuga* and *P. velascoi*) should be considered different species under the morphological, biological, and phylogenetic species concepts.

Biogeographical considerations and divergence times

The phylogenetic relationships of the four basal clades remain unclear since they were joined in an unresolved polytomy. It is not excluded that this could be a hard polytomy (Walsh *et al.* 1999) if the radiation of the four clades happened in a short period of time. This seems to be supported by the very similar mean p-distances between them. However, from the beginning of the *Pyrenaearia* diversification to the present time, several lineages could have become extinct, although fossil evidence has not been reported. Missing evolutionary intermediates can indeed lead to phylogenetic artifacts, including unresolved nodes (Emerson 2002, Holland and Hadfield 2004).

A molecular clock is required for estimating divergence times. Unfortunately, the evolutionary rate of COI, 16S, or ITS-1 sequences of *Pyrenaearia* is unknown. Evolutionary rates of mtDNA are often estimated at 2% pairwise sequence divergence per million years (My) for invertebrates (DeSalle *et al.* 1987). Yet, faster clocks have been postulated in some terrestrial snails (Thomaz *et al.* 1996), so that mtDNA evolutionary rates of terrestrial gastropods tentatively vary between 1% and 10% per My (Thomaz *et al.* 1996, Douris *et al.* 1998, Chiba 1999, Hayashi and Chiba 2000). Sometimes, 2-5% sequence divergence per My has been tentatively used for several helicoids (Thomaz *et al.* 1996, Pfenninger *et al.* 2003). Hayasi and Chiba (2000) estimated a divergence rate of 10% for 16S in a bradybaenid using more reliable data than previous speculations. The mean values of the uncorrected average p-distances among basal clades were 8.5-9.8% for COI and 5.2-7.2% for 16S. Using a fast rate of 10% per My, the four clades would have separated during the Pleistocene (0.5-1.0 My). However, applying a more conventional divergence rate of 2% would imply that separation of the four clades could be dated between 2.6 and 4.9 My ago, in the Pliocene. Consequently, the split of the four major lineages in *Pyrenaearia* probably predated the Pleistocene glaciations as also postulated for other animal groups (Hewitt 1996, Taberlet *et al.* 1998). The genetic distances of mtDNA genes between clade 3a and the other subgroups of clade 3 (b-f) were similar to those indicated for the four basal clades (7.7%, 6.9% for COI, 16S) and probably predated the Pleistocene glaciations as well. The mean values of the average pairwise

genetic distances among the clades 3b-f ranged between 2.8% and 5.7% for COI and 2.2%-3.3% for 16S. Hence, the separation of clades 3b-f could be dated during Pleistocene glaciations [from Günz on, 600-500 thousand years (ky)], when considering the fast molecular rate of 10% per My.

Consequently, *Pyrenaearia* speciation within the four main groups is supposed to have occurred during cooling and warming cycles of the Pleistocene, which were particularly intense during the last 600 ky. During Pleistocene cooling periods, the high-altitude adapted populations had to migrate from their warm period refuges on the top of the mountains to lower altitudes in the valleys of the Cantabro-Pyrenaean region and the Ebro valley as a consequence of the permanent ice coverage of the mountains. In some cases, this downward migration was probably followed by expansions of the distributional ranges of cold-adapted species, resulting in possible secondary contacts. The presence of *Pyrenaearia velascoi* in the Cantabrian mountains seems to be the result of one of such expansion events that occurred probably during the last glaciation (Würm, 120-18 ky). This expansion of *P. carascalensis s.l.* to the west would have resulted in the secondary contact between the pyrenaean *P. carascalensis s.l.* and the cantabrian *P. cantabrica* lineages, but the previous allopatric speciation of these two lineages must have early enough to build up reproductive isolation.

The most recent speciation process began with the last deglaciation phase, starting ca. 14,000 ky BP, resulting in the shrinkage of the distribution range of cold-adapted species. This may have led to, for example, the isolation and subsequent genetic differentiation between *Pyrenaearia carascalensis s.s.* and *P. velascoi*. The small population of *P. carascalensis* living in Aspe valley (=*P. transfuga* morphospecies) could be a relict population surviving at low altitudes or may have been established by passive transport (heavy wind, rainstorms, birds) as was described in some other terrestrial molluscs (Gittenberger *et al.* 2006). The separation of two populations of *P. navasi*, one restricted to high altitudes (above 2000 m), the other sheltered within deciduous forests in the same mountainous system seems to also postdate the Last Glacial Maximum. The resolution of all these and some other questions needs more field and laboratory research.

Several workers have demonstrated that terrestrial molluscs are valuable organisms to investigate evolutionary processes in species with poor dispersal abilities (Davison and Clarke 2000, Hausdorf and Henning 2004, Schilthuizen *et al.* 2004, 2006, Uit de Weerd 2004, Ketmaier *et al.* 2006). The land snail genus *Pyrenaearia* is a polytypic group of closely related taxa that could constitute a good model organism for molecular phylogenetic studies. The existence of several taxa, which are confined to high-altitudes and, consequently, are intensely affected by insularity phenomena, makes these organisms suitable for the study of allopatric and/or peripatric speciation processes of isolated lineages. Moreover, the presence of some species living at high altitudes in close vicinity to other species restricted to the valleys within the same mountainous system also makes them a good model to study parapatric speciation. The effects of geo-historical climate changes on high-altitude species is poorly understood (Haubrich and Schmitt 2007). The genus *Pyrenaearia* could also represent an ideal group for the study of the effects of cyclic climate changes on geographic distribution of gene lineages and population structure in a group containing several taxa with subalpine affinities.

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Appendix 1. Species abbreviations, geographical coordinates (using Spanish grid references), and GenBank accession numbers for all the sequences of *Pyrenaearia*. Asterisks (*) and bold indicate topotypes.

Abbreviation	Localities-U.T.M.	GenBank access number of COI	GenBank access number of 16S rRNA	GenBank access number of ITS1
P. carascalopsis*-01	Port d´Salau (Lleida) 31TCH4733	EU310004	EU310083	EU310162
P. carascalopsis-02	Alto Aneu (Lleida) 31TCH4133	EU310005	EU310084	EU310163
P. esserana*-01	La Renclusa (Huesca) 31TCH0727	EU310006	EU310085	EU310164
P. esserana-02	Port de Viella (Lleida) 31TCH1624	EU310007	EU310086	EU310165
P. esserana-03	Port de Viella (Lleida) 31TCH1624	EU310008	EU310087	EU310166
P. parva*-01	Pedraforca (Barcelona) 31TCG9377	EU310009	EU310088	EU310167
P. parva*-02	Pedraforca (Barcelona) 31TCG9377	EU310010	EU310089	EU310168
P. parva-03	Sierra de Cadí (Barcelona) 31TCG9582	EU310011	EU310090	EU310169
P. parva-04	Sierra de Cadí (Barcelona) 31TCG9582	EU310012	EU310091	EU310170
P. organiaca*-01	Congost d´Organya (Lleida) 31TCG6377	EU310013	EU310092	EU310171
P. organiaca*-02	Congost d´Organya (Lleida) 31TCG6377	EU310014	EU310093	EU310172
P. navasi navasi*-01	Moncayo (Zaragoza) 30TWM9726	EU310015	EU310094	EU310173
P. navasi navasi*-02	Moncayo (Zaragoza) 30TWM9726	EU310016	EU310095	EU310174
P. navasi sylvatica*-03	Moncayo (Zaragoza) forest 30TWM9829	EU310017	EU310096	EU310175
P. navasi sylvatica*-04	Moncayo (Zaragoza) forest 30TWM9829	EU310018	EU310097	EU310176
P. molae*-01	Mola de Colldejou (Tarragona) 31TCF2153	EU310019	EU310098	EU310177
P. molae*-02	Mola de Colldejou (Tarragona) 31TCF2153	EU310020	EU310099	EU310178
P. cotiellae*-01	Circo de Armeña (Huesca) 31TBH8010	EU310021	EU310100	EU310179
P. cotiellae*-02	Circo de Armeña (Huesca) 31TBH8010	EU310022	EU310101	EU310180
P. cotiellae-03	Collado de Sahún (Huesca) 31TBH8616	EU310023	EU310102	EU310181
P. cotiellae-04	Collado de Sahún (Huesca) 31TBH8616	EU310024	EU310103	EU310182
P. carascalensis-01	Muntaya de Casamanya (Andorra) 31TCH8215	EU310025	EU310104	EU310183
P. carascalensis-02	Muntaya de Casamanya (Andorra) 31TCH8215	EU310026	EU310105	EU310184
P. carascalensis-03	Port Boucharo. Gavarnie (Francia) 30T YN4032	EU310027	EU310106	EU310185
P. carascalensis-04	Port Boucharo. Gavarnie (Francia) 30T YN4032	EU310028	EU310107	EU310186
P. carascalensis-05	Pico Anie (Navarra) 30TXN8555	EU310029	EU310108	EU310187
P. carascalensis-06	Pico Anie (Navarra) 30TXN8555	EU310030	EU310109	EU310188
P. carascalensis -07	Pico Anie (Navarra) 30TXN8555	EU310031	EU310110	EU310189
P. carascalensis-08	Portalet (Huesca) 30TYN1042	EU310032	EU310111	EU310190
P. carascalensis-09	Pico Anie (Navarra) 30TXN8555	EU310033	EU310112	EU310191
P. transfuga*-01	Pont d´Esquit (Francia) 30TXN9559	EU310034	EU310113	EU310192
P. transfuga*-02	Pont d´Esquit (Francia) 30TXN9559	EU310035	EU310114	EU310193
P. velascoi*-01	Aldamin (Bizkaia) 30TWN1865	EU310036	EU310115	EU310194
P. velascoi*-02	Aldamin (Bizkaia) 30TWN1865	EU310037	EU310116	EU310195
P. velascoi-03	Gorbea frente Aldamin (Bizkaia) 30TWN1865	EU310038	EU310117	EU310196
P. velascoi-04	Txindoki (Gipuzkoa) 30TWN7463	EU310039	EU310118	EU310197
P. velascoi-05	Txindoki (Gipuzkoa) 30TWN7463	EU310040	EU310119	EU310198
P. velascoi-06	Aitzgorri (Gipuzkoa) 30TWN5456	EU310041	EU310120	EU310199
P. velascoi -07	Monte Altxueta (Navarra) 30TWN8456	EU310042	EU310121	EU310200
P. velascoi-08	Aitzgorri (Gipuzkoa) 30TWN5456	EU310043	EU310122	EU310201
P. velascoi-09	Peña Lekanda (Bizkaia) 30TWN1668	EU310044	EU310123	EU310202
P. velascoi-10	Peña Lekanda (Bizkaia) 30TWN1668	EU310045	EU310124	EU310203
P. velascoi-11	Beriain (Navarra) 30TWN8349	EU310046	EU310125	EU310204
P. velascoi-12	Beriain (Navarra) 30TWN8349	EU310047	EU310126	EU310205
P. cantabrica*-01	Caldas de Oviedo (Asturias) 30TTP6300	EU310048	EU310127	EU310206
P. cantabrica*-02	Caldas de Oviedo (Asturias) 30TTP6300	EU310049	EU310128	EU310207
P. cantabrica-03	Peña Lekanda (Bizkaia) 30TWN1668	EU310050	EU310129	EU310208
P. cantabrica-04	Txarlazo (Bizkaia) 30TVN9659	EU310051	EU310130	EU310209
P. cantabrica-05	Txarlazo (Bizkaia) 30TVN9659	EU310052	EU310131	EU310210

Appendix 1. (continued)

Abbreviation	Localities-U.T.M.	GenBank access number of COI	GenBank access number of 16S rRNA	GenBank access number of ITS1
P. cantabrica-06	Beriain (Navarra) 30TWN8349	EU310053	EU310132	EU310211
P. cantabrica-07	Beriain (Navarra) 30TWN8349	EU310054	EU310133	EU310212
P. cantabrica-08	Beriain (Navarra) 30TWN8349	EU310055	EU310134	EU310213
P. cantabrica-09	Proaza (Asturias) 29TQH4192	EU310056	EU310135	EU310214
P. cantabrica-10	Langro-Pto Tarna (Asturias) 30TUN1281	EU310057	EU310136	EU310215
P. cantabrica-11	Langro-Pto Tarna (Asturias) 30TUN1281	EU310058	EU310137	EU310216
P. cantabrica-12	Aldamin (Bizkaia) 30TWN1865	EU310059	EU310138	EU310217
P. cantabrica-13	Poncebos (Asturias) 30TUN5191	EU310060	EU310139	EU310218
P. cantabrica-14	Poncebos (Asturias) 30TUN5191	EU310061	EU310140	EU310219
P. cantabrica-15	Caranga-Quirós (Asturias) 29TQH4089	EU310062	EU310141	EU310220
P. cantabrica-16	Caranga-Quirós (Asturias) 29TQH4089	EU310063	EU310142	EU310221
P. cantabrica-17	Cordiñanes-Caín (Asturias) 30TUN4582	EU310064	EU310143	EU310222
P. cantabrica-18	Cordiñanes-Caín (Asturias) 30TUN4582	EU310065	EU310144	EU310223
P. cantabrica-19	Desfiladero de los Beyos (Asturias) 30TUN3088	EU310066	EU310145	EU310224
P. cantabrica-20	Desfiladero de los Beyos (Asturias) 30TUN3088	EU310067	EU310146	EU310225
P. cantabrica-21	Anboto (Bizkaia) 30TWN3270	EU310068	EU310147	EU310226
P. cantabrica-22	Anboto (Bizkaia) 30TWN3270	EU310069	EU310148	EU310227
P. daanidentata*-01	Hoyo del Burro (León) 30TUN4082	EU310070	EU310149	EU310228
P. daanidentata-02	Canal del Perro (León) 30TUN4082	EU310071	EU310150	EU310229
P. oberthueri-01	Vega Urriello (Asturias) 30TUN5285	EU310072	EU310151	EU310230
P. oberthueri-02	Vega Urriello (Asturias) 30TUN5285	EU310073	EU310152	EU310231
P. oberthueri-03	Vega Urriello (Asturias) 30TUN5285	EU310074	EU310153	EU310232
P. oberthueri-04	Vega Urriello (Asturias) 30TUN5285	EU310075	EU310154	EU310233
P. oberthueri-05	Peña Santa Ana (León) 30TUN5282	EU310076	EU310155	EU310234
P. oberthueri-06	Peña Santa Ana (León) 30TUN5282	EU310077	EU310156	EU310235
P. poncebensis*-01	Poncebos (Asturias) 30TUN5191	EU310078	EU310157	EU310236
P. poncebensis*-02	Poncebos (Asturias) 30TUN5191	EU310079	EU310158	EU310237
P. schaufussi*-01	Urdón (Asturias) 30TUN6791	EU310080	EU310159	EU310238
P. schaufussi*-02	Urdón (Asturias) 30TUN6791	EU310081	EU310160	EU310239
Hygromia limbata	Pagasarri (Bizkaia) 30TWN0485	EU310082	EU310161	EU310240
Iberus gualtieranus	Huercal de Almería (Almería) 30SWF4880	AY928568	AY928596	EU446026